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TITLE: Targeting Neuronal-like Metabolism of Metastatic Tumor Cells as a Novel Therapy for Breast Cancer Brain Metastasis

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14. ABSTRACT
During this report period, we conducted both intravital imaging and whole tissue clearing based imaging to dissect the interaction between tumor cell and its brain metastatic microenvironment. We have successfully expanded GFAP-GFP mouse line (brain astrocyte specific) and performed preliminary testing on the intravital imaging capability. In addition, we have streamlined and optimized tissue-clearing pipeline. We are able to multiplex staining multiple tissue markers in situ, including brain astrocytes, tumor cell, proliferating cell (Edu) and blood vessel. We have conducted 3D reconstruction of brain metastasis landscape. We will conduct detailed quantifications of large 3D imaging dataset we have obtained. Larger scale in vivo experiments will be conducted in the coming year.

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1. Introduction……

Among all breast cancer metastatic replaces, 30% of breast cancer-associated fatalities being attributable to breast cancer brain metastasis. Patients with brain metastatic relapse have a median survival of less than one year. Currently, no effective drug treatments are available for brain metastasis. In this proposal, we hypothesize: interactions with reactive brain astrocytes and transcriptome reprogramming during metastatic evolution of tumor cells represent intriguing therapeutic targets for brain metastasis treatment. We will take state-of-the-art imaging approaches to study the interaction between tumor cell and brain metastatic environment. We will determine the functional importance of key neuronal-like changes during metastatic evolution and target metastatic colonization of the brain with BBB-permeable neurological drugs. In this project, we propose two specific aims to explore the functional importance of the early metastatic evolution and the feasibility of targeting metastatic evolution by repurposing neurological drugs. **Aim 1:** Study the spatial and temporal interactions between brain astrocytes and metastatic tumor cells *in situ*. **Aim 2:** Pre-clinically investigate the therapeutic efficacy of co-targeting glutamate receptors signaling and breast cancer driver genes.

2. Keywords……

Breast cancer, brain metastasis, metastatic outgrowth, brain intravital imaging, tissue clearing, glutamate signaling.

3. Accomplishments……..……..

What were the major goals of the project? What was accomplished under these goals?
Based on our original SOW, during the first funding year, we aim to conduct research as proposed in the original Aim 1. For your easy reference, the original milestones in SOW are listed below as font *italics 11pt*.

Major Task 1 Aim 1.1. Milestone(s) to achieve:
1) Local IRB/IACUC Approval

Progresses: We have applied IACUC and obtained ACURO approval on 10/6/2015 on animal experiments proposed in this proposal.

2) Establish GFAP.GFP colony
3) Intravital imaging of metastasis early colonization (3 repeats) and data analyses

Progresses: We have started pilot experiment for intravital imaging of early-disseminated tumor cells. We perfected cranial window surgery in the past 6 months and observed less surgery-induced bleeding/inflammation. We first labeled the blood vessel by i.v. injection of TexasRed-dextran and then measured the average size diameter of the blood vessel that contains tumor cells (Fig. 1a-c). Disseminated tumor cells exhibited a highly diverse morphology and high degree of membrane deformability inside the blood vessel. On average, tumor cells colonized in brain micro vessels which have vessel diameter between 7.5 -12.5 μm (Fig. 1c).

Since we received ACURO approval, we purchased two pairs of *FVB/N-Tg(GFAP.GFP)*$^{14Mes/J}$ mice from Jax laboratory to start the mouse colony. This stain of mouse specifically labels astrocytes (GFAP-expressing) with GFP. Therefore, it allows us to monitor the dynamics changes of astrocytes using intravital imaging. We have established mouse colony and we are expanding this line of animals. Since we will need a sufficient number of age-matched animals for each experimental group, we are accumulating experimental mice currently. Up to today, we conducted limited feasibility experiments to validate the usability of *FVB/N-Tg(GFAP.GFP)*$^{14Mes/J}$ mice. We have confirmed genotype of the F1 generation of mouse colony.

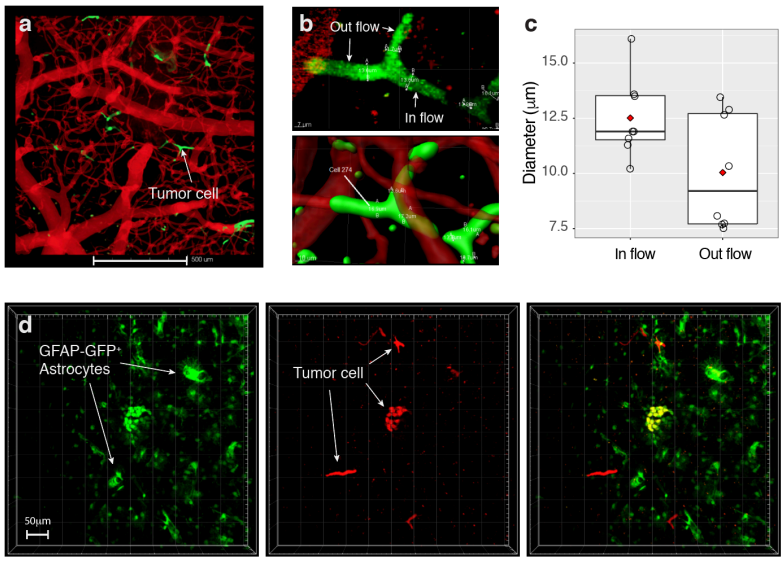

Figure 1 Characterization of intravital imaging of brain metastasis. (a) 3D view of disseminated tumor cell (GFP+) and blood vessel staining (TexasRed Dextran). Bar = 500μm. (b) Measurements of blood vessel confinement of single disseminated tumor cell. Top panel: raw image; bottom panel: 3D reconstructed image. (c) Quantifications of blood vessel confinement. (d) Intravital imaging of metastatic tumor cells in the brain at day 1 of metastasis dissemination. Green: GFAP-GFP+ astrocytes; Red: Td-tomato Red labeled tumor cells.

After induced tumor formation by intracardiac injection, we collected mouse brain and monitored the GFP-expression in brain astrocytes (Fig. 1d). Interestingly, we observed tumor cell dissemination into brain and blood vessels was covered by brain astrocytes (Fig 1d). Due to the limited mice currently available in our colony, we did not perform full-scale time-lapse imaging experiments as planned. We will conduct intravital experiments in the coming funding year. Instead, in the past funding year, we performed Aim 1.2 in parallel and have made significant progresses in whole tissue imaging (see below).

Major Task 1 Aim 1.2. *Milestone(s) to achieve:*
1) Collect brain metastasis samples from multiple animal cohorts

Progresses: We injected MDA-MB-231 cells into immunodeficient Rag1 -/- to establish early metastases and collected the brain at different days post-injection. It generally takes around 30-60 days for MDA-MB-231 model to form brain metastasis in Rag 1 -/- mice. We have successfully finished the first round of in vivo experiment and collected metastases-bearing brains for imaging analysis (see below). Future in vivo experiments (biological repeats) will be conducted in the coming funding year.

2) Clear tissue specimen
3) Major markers are IF stained
4) 2-photon deep tissue imaging are performed
5) Reconstruction of multiplexed 3D brain metastasis and data analyses

Progresses: We collected mice brain from the different stages of brain metastasis development (early stage ~2 weeks, early outgrowth stage ~3 weeks and late outgrowth stage ~4-6 weeks post

injection). After perfusion of tumor-bearing brains with PBS and fixatives (4% PFA), we performed tissue optical clearing (CLARITY/CUBIC) based on established protocols as we did in our preliminary studies. We streamlined a whole tissue clearing, staining, imaging and successfully imaged the whole mouse brain with full antibody penetration. Tissue clearing and refractive index matching rendered the brain lipid-free and optically transparent. As shown in Fig. 2, using a special 8-mm working distance objective lens developed for tissue clearing, we achieved an approximate five-fold increase in imaging depth from ~500 μm (Fig. 2a, left) to ~3000 μm (Fig. 2b, right). We also tested multiplexed staining for proliferative nuclei, metastatic tumor cells, and brain micro-environmental components, particularly astrocytes (anti-GFAP staining). After staining and imaging, our approach allowed us to 3D co-registration of multiple metastatic landscape components with high spatial resolution on a global scale of dimensions up to approximately 4000μm x 4000μm x 3000μm (Fig. 2b). We are excited to report that this exponential increase of data content will not only enable us to reconstruct the brain metastasis landscape in 3D, but also provides new, exceptionally accurate perspectives on phenotypic heterogeneity of metastasis progression, such as the highly irregular tumor morphology that is masked in two-dimensional images (Fig. 2c). We were able to glean detailed information from large, continuous tissue structures at high 3D resolution with the cellular resolution, such as one single extravasated metastatic cell (Fig. 2d) and subcellular details, such as dividing nuclei using EdU staining (Fig. 2e). In the coming funding year, we will continue using our unique 3D imaging approach to systematically quantify the metastasis phenotype and interactions between tumor cell and its brain microenvironment.

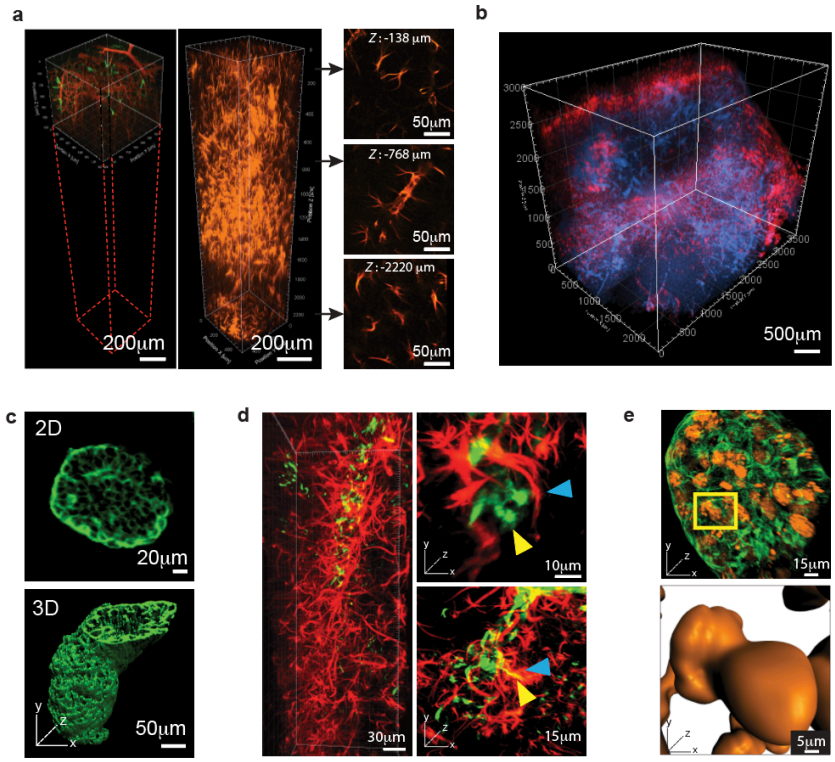

Figure 2 3D Whole Tissue Imaging of the Brain Metastasis Landscape with Molecular Resolution. (a) Comparison of multiphoton-imaging depth without (left) or with (right) optical tissue clearing. 2D slices are extracted from indicated depths in the tissue-cleared z-stack to demonstrate image quality at various imaging depths. (b) Multiphoton image of 3D global view (2500μm x 3500μm x 3000μm) of optically cleared mouse brain with multiple MDA-MB-231.Br-derived metastases. Red: anti-GFAP; blue: DAPI. (c) 2D multiphoton image (top) and 3D surface generated image (bottom) of PNA.Met1 brain metastases stained with anti-cytokeratin 8 (K8). (d) 3D multiphoton image of astrocytes (GFAP, red) of the blood brain barrier (left) and astrocytes interaction with MDA-MD-231.Br metastatic cells (right). Blue arrows point to astrocytes, and yellow arrows point to metastatic cells. (e) 3D multiphoton image of PNA.Met1 brain metastasis (K8, green) and EdU-tagged nuclei (orange) (left) and enlarged view of dividing nuclei (right).

What opportunities for training and professional development has the project provided?
This project provides a unique multidisciplinary training opportunity for my trainees who traditionally trained as classic cell/cancer biologists. This imaging-heavy project allows trainees

to obtain advanced skill set in whole tissue imaging and multiphoton microscopy. Analyzing TB-level imaging dataset also provide a unique training aspect of "big-data" system cancer biology.

How were the results disseminated to communities of interest?
We have participated community outreach activity at Notre Dame, e.g. Notre Dame Day, to disseminate some of our exciting results to lay public. We also used our tissue clearing and 3D imaging as powerful tools to engage high school students who are interested in STEM career path. For instance, we have recently hosted an on-site science visit for Penn High School students.

What do you plan to do during the next reporting period to accomplish the goals?

In the next funding year, we will continue develop our imaging platform to obtain insight on molecular mechanisms of metabolic shifting. We aim to achieve the following goals:

1) Perform intravital imaging on astrocytes-tumor cell interaction in real-time.
2) Develop and validate whole tissue staining of glutamate/glutamine receptors.
3) Develop and validate image data analysis pipeline to achieve precise and unbiased quantification of 3D image data sets.
4) Start to explore the functional importance of metabolic genes in regulating brain metastasis success.

4. Impact……...……

What was the impact on the development of the principal discipline(s) of the project?
Using whole tissue and intravital imaging approach to study metastasis is highly innovative. To our knowledge, we are the first group that applies this methodology in the cancer research field. As the cancer research shifts from "one-gene a time" approach to a global unbiased view of cancer as an interconnected tissue, our whole tissue 3D imaging approach provide an unique perspective to traditional cancer research field.

What was the impact on other disciplines?
To analyze the big 3D imaging dataset effectively, we are collaborating with our collaborators in the computational engineering department who are specialized in imaging progressing to develop robust image segmentation/analysis methods. This collaborative effort will lead to more powerful tools for traditional biologist in cancer biology and related field.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. Changes/Problems....….……

Nothing to report

6. Products…….……..……

We are in the process of summarizing imaging experiment into manuscript entitled: "An Integrative Platform for Three-dimensional Quantitative Analysis of Spatially Heterogeneous Metastasis Landscapes". This award is acknowledged.

7. Participants & Other Collaborating Organizations……

What individuals have worked on the project?

Name:	*Siyuan Zhang*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*A-1276-2014*
Nearest person month worked:	*3*
Contribution to Project:	*As the PI of this project, Dr. Zhang oversees the project design and data interpretation.*
Funding Support:	*DoD (this award)*

Name:	*Patricia Skallos*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*12*
Contribution to Project:	*Patricia's thesis work primarily focuses on this project. She plays a major role in conducting in vitro and in vivo biology experiments and bioinformatics analysis.*
Funding Support:	*Partially funded by DoD (this award) and Walther Foundation for Cancer Research (pre-doctoral fellowship)*

Name:	*Ian Guldner*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*3*
Contribution to Project:	*Ian primarily focues on developing tissue imaging pipeline and perform imaging data analysis.*
Funding Support:	*Partially funded by DoD (this award) and departmental teaching assistantship.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements……

Nothing to report

9. Appendices……

Nothing to report